A self-replicating peptide nucleic acid

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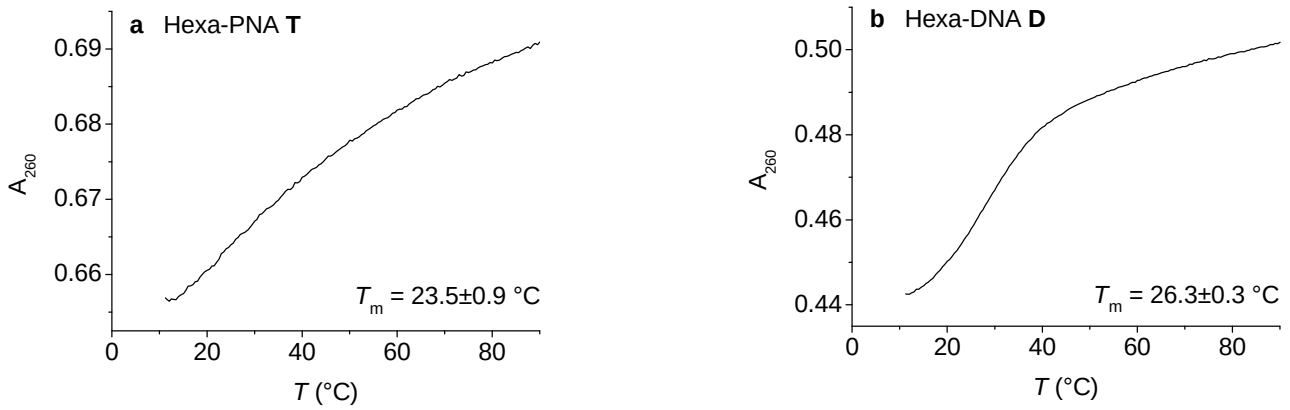
Table of contents

1 Analytical characterization of hexa-PNA T

1.1 General methods

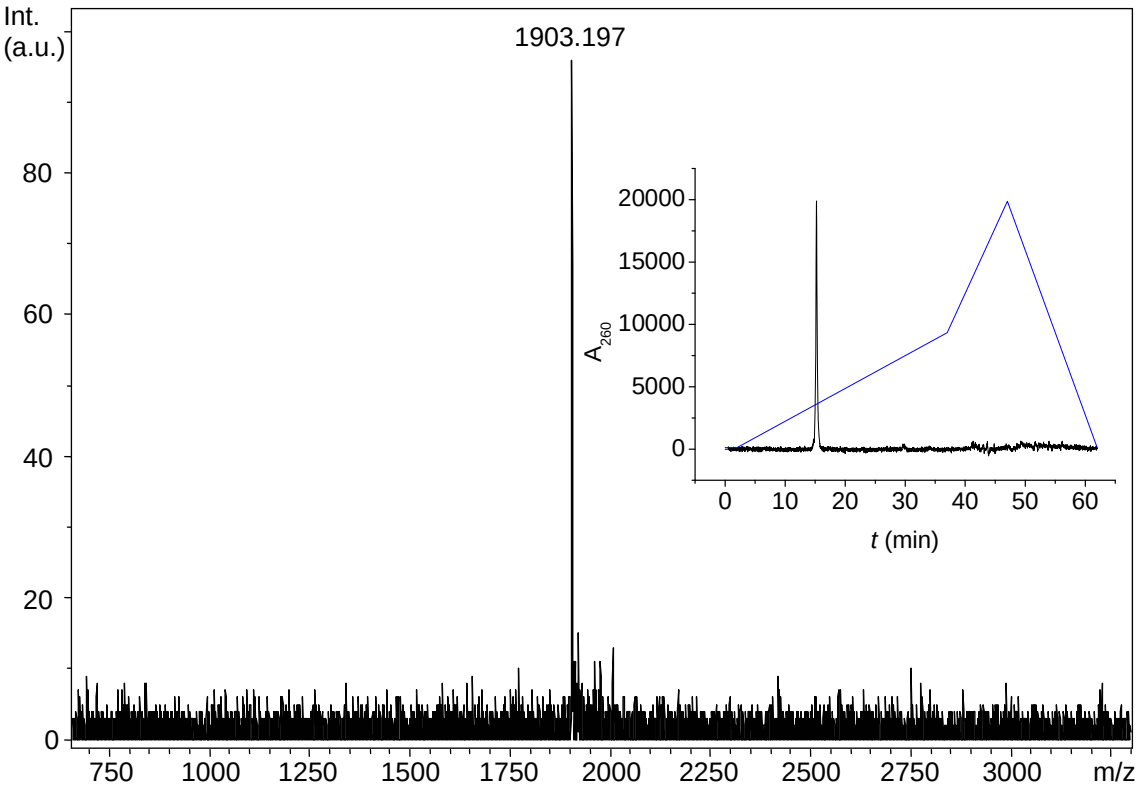
Freeze drying. *Christ* Alpha 1-2. **Mass spectrometry.** MALDI-TOF spectra were recorded on a *Bruker daltonics* autoflex using 2',4',6'-trihydroxyacetophenone as matrix. HR-ESI-MS was run on a *Thermo Scientific* LTQ-Orbitrap XL. **Semi-prep. HPLC.** 250 × 10 mm *Supelco* Discovery BIO Wide Pore C18 5 µm; flow 3 ml/min; *T* 55 °C. **Synthesis.** The synthesis and characterization of F-PNA **A** and PNA **B** was reported elsewhere (26). Hexa-PNA **T** was isolated from preliminary experiments without initially added template using semi-prep. RP-HPLC. All other chemicals were used as delivered, usually in p.a. quality. **UV.** *Varian* Cary 1E.

1.2 Melting curves



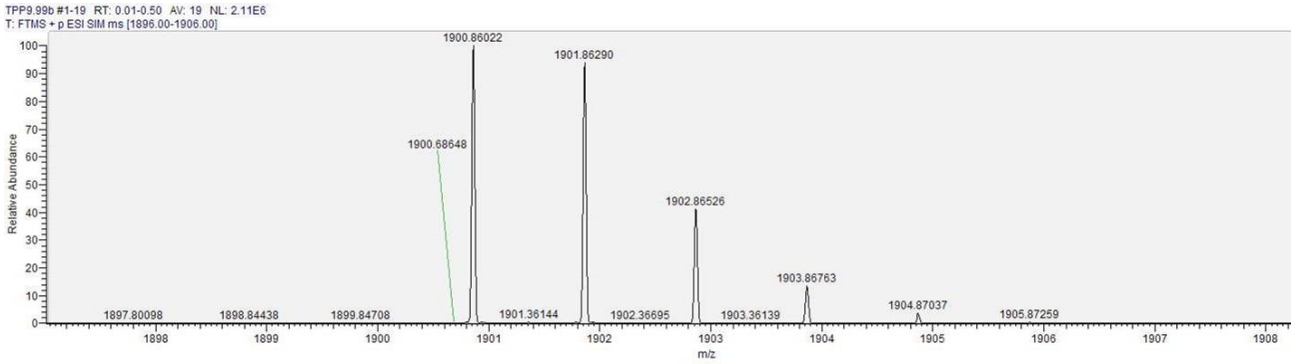
Supplementary Figure S1. UV melting profiles at 260 nm. **a,** Hexa-PNA **T** (^{N}cfgcag^{C}). **b,** Comparable hexa-DNA **D** (5'-CTGCAG-3'). Conditions: 0.25 M sodium phosphate pH 7, 0.1 M NaCl, heating: 1 °C/min.

1.3 RP-HPLC and MALDI-TOF-MS



Supplementary Figure S2. MALDI-TOF mass spectra and RP-HPLC plot of hexa-PNA **T** (therein). Conditions: LP, THAP (MS); 55 °C, 1 ml/min (HPLC). Gradient: 2 min 5 %B, 5 → 40 %B in 35 min, 40 → 80 %B in 10 min, 80 %B → 5 %B in 15 min. MALDI-MS (*m/z*): $[M + 3\ H]^+$ calcd. for $C_{79}H_{110}F_2N_{37}O_{18}^+$, 1902.8797; found, 1903.197.

1.4 HR-ESI-MS



Supplementary Figure S3. HR-ESI mass spectra of hexa-PNA **T**. ESI-HRMS (*m/z*): $[M + 5\ H]^+$ calcd. for $C_{79}H_{112}F_2N_{37}O_{18}^+$, 1904.8954; found, 1904.8704; $[M + 4\ H]^+$ calcd. for $C_{79}H_{111}F_2N_{37}O_{18}^+$, 1903.8875; found, 1903.8673; $[M + 3\ H]^+$, calcd. for $C_{79}H_{110}F_2N_{37}O_{18}^+$, 1902.8797; found, 1902.8653; $[M + 2\ H]^+$, calcd. for $C_{79}H_{109}F_2N_{37}O_{18}^+$, 1901.8719; found, 1901.8629; $[M + H]^+$, calcd. for $C_{79}H_{108}F_2N_{37}O_{18}^+$, 1900.8641; found, 1900.8602.

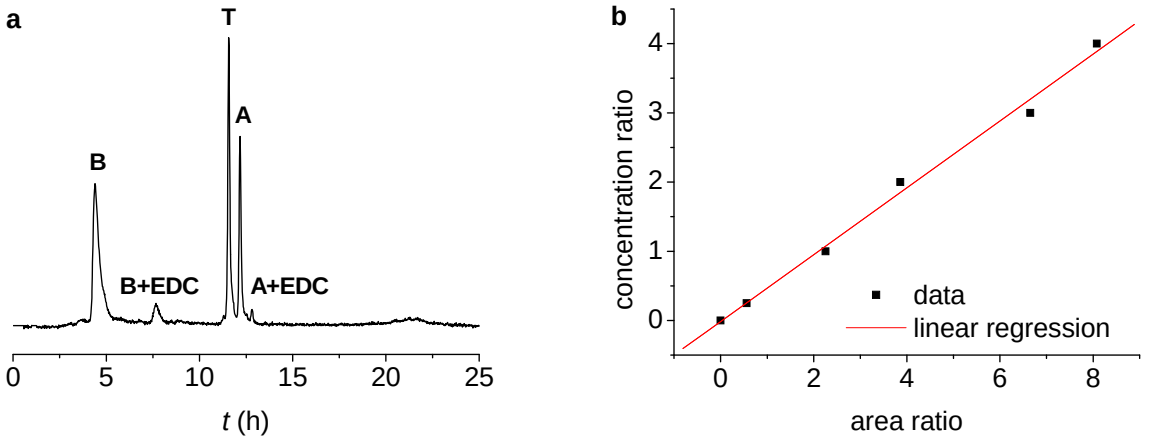
2 HPLC analysis and calibration

2.1 HPLC analysis.

Analytical HPLC (*Beckman* Gold system: 126 Solvent Module, 168 Detector) was run on a *Supelco* Ascentis RP Amide column (250×4.6 mm, 5 µm particle size) at 55 °C (*Jetstream* 2 Plus Column-Thermostat) with a linear gradient of 5-35 % of solvent B (0.1 % TFA in acetonitrile) (0-15 min) in solvent A (0.1% TFA in deionized and dist. H_2O) at a flow rate of 1 ml/min.

2.2 HPLC calibration

Concentrations of PNA **A** and **T** were calculated from the respective HPLC areas by means of an internal, ratio based calibration because both compounds gave sharp signals with comparable retention times (Supplementary Fig. S3). To this end, a calibration curve was recorded that correlated the area ratio with the concentration ratio of both compounds (Supplementary Fig. S3). Thus, the results are independent of changes in sample concentration or volume errors during sampling, dilution, and injection. Empirically, we found a linear relationship (equation (S1)).



Supplementary Figure S4. a, Representative HPLC plot at λ = 260 nm. All peaks were isolated and analyzed by MALDI-TOF-MS. **A+EDC** is an EDC adduct of **A** with unknown constitution. **B+EDC** is presumably a guanidinium structure formed by addition of N-terminal amino to EDC. **b,** Calibration curve for converting the area ratio into the corresponding concentration ratio. Fit parameter: $R^2 = 0.9936$.

$$f(x) = (-0.02 \pm 0.09) + (0.48 \pm 0.02) \times x. \quad \text{(S1)}$$

Assuming that tri-PNA **A** reacts solely to hexa-PNA **T** gives:

$$[\mathbf{T}] + [\mathbf{A}] = [\mathbf{T}]_0 + [\mathbf{A}]_0 \tag{S2}$$

[**T**] = concentration of **T** at time t

[**A**] = concentration of **A** at time t

$[\mathbf{T}]_0$ = initial concentration of **T**

$[\mathbf{A}]_0$ = initial concentration of **A**

Thus, the concentration ratio κ can be expressed by:

$$\kappa = \frac{[\mathbf{T}]}{[\mathbf{A}]} = \frac{[\mathbf{T}]}{[\mathbf{A}]_0 + [\mathbf{T}]_0 - [\mathbf{T}]} \tag{S3}$$

Finally, we may rewrite equation (S3) to calculate the concentration of **T** at time t:

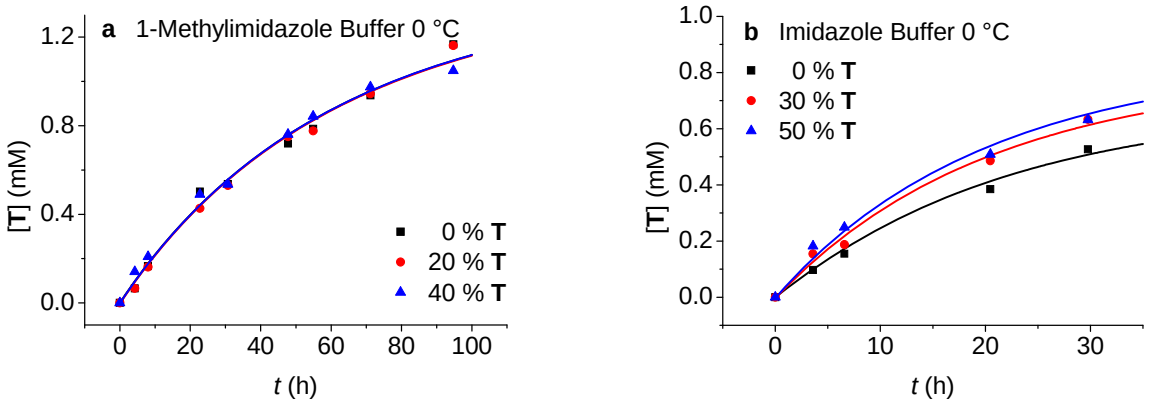
$$[\mathbf{T}] = \frac{\kappa([\mathbf{T}]_0 + [\mathbf{A}]_0)}{\kappa + 1} \tag{S4}$$

This calibration method can also be used if detectable amounts of EDC adduct **A+EDC** are formed during the reaction: In this case [**A**] has been replaced by [**A**] + [**A+EDC**] in equations (S1)-(S4).

3 PNA self-replication assays

Reaction solutions were prepared in 500 µl polypropylene sample tubes (Sarstedt) from a 10 mM stock solution of tri-PNAs **A** and **B**. To start the reaction, the same volume of a solution of EDC, nucleophilic catalyst, and salt in the respective buffer was added. The mixtures were immediately vortexed, centrifuged, and split into a series of sample tubes which, where required, contained lyophilized hexa-PNA **T**. The resulting solutions were cooled (MJ Research MiniCycler PTC-150) or kept at r.t. At appropriate times, 0.5 µl reaction solution was added to 500 µl of an aqueous solution containing 5 % MeCN and 0.1 % TFA. After mixing and vortexing, the resulting solution was immediately analyzed by RP-HPLC or kept at r.t. Storage at r.t. for several days had no effect on sample composition.

The figures following below show experimental data points and theoretical curves from *SimFit* runs based on equations (1)-(3) as the reaction model (model B). In order to detect self-replication, the data points were also approximated using equations (1) and (3) thus omitting a self-replicating pathway (model A). In addition to ε, a significantly better agreement of the experimental data with model B, as defined by the root mean square (RMS), was consequently taken as a further indication for self-replication. In addition to the RMS, the reliability of the results was justified by the off-diagonal elements of the covariance matrix, where absolute values significantly below 1 indicate an acceptable independency between the parameters. In many cases experiments are shown which compare the results obtained at $T = 10$ °C (solid curves) with those at room temperature (dashed curves) where template effects are less pronounced to negligible. Here, due to the absence of measurements involving template seeding the fittings are generally less reliable. These experiments nevertheless allowed to identify conditions for efficient template independent ligation. Some catalysts obviously enhance template free ligation while suppressing the template directed pathway (see Supplementary Fig. S7). A detailed mechanistic rationale is difficult to provide based on the kinetic screening data underlying our study. We are thus not able to distinguish whether the effect of such catalysts is due to a thermodynamic destabilization of termolecular complexes or due to a disfavourable orientation of the catalytic leaving group.



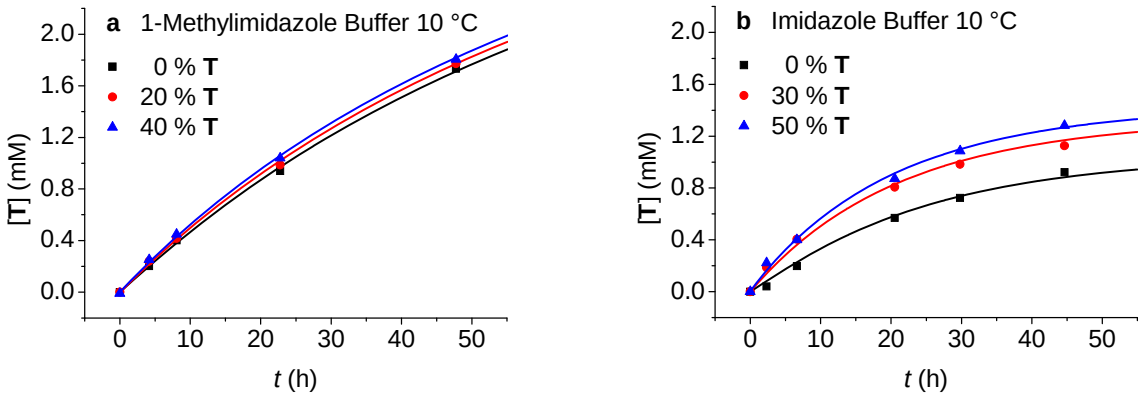
Supplementary Figure S5. Kinetics of PNA self-replication involving two different imidazole buffers at $T = 0$ °C (model B). Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, a: 0.4 M 1-methylimidazole pH 7.5, b: 0.4 M imidazole pH 7.5, 0 °C, 25 µl scale. a: $\mathrm{cov}(k_1, k_2) = -0.571$, $\mathrm{cov}(k_1, k_3) = 0.590$, $\mathrm{cov}(k_2, k_3) = 0.263$, $\mathrm{cov}(k_1, c_1) = -0.123$, $\mathrm{cov}(k_1, c_2) = -0.073$, $\mathrm{cov}(k_2, c_1) = -0.178$, $\mathrm{cov}(k_2, c_2) = -0.478$, $\mathrm{cov}(k_3, c_1) = -0.189$, $\mathrm{cov}(k_3, c_2) = -0.252$, and $\mathrm{cov}(c_1, c_2) = 0.235$. b: $\mathrm{cov}(k_1, k_2) = -0.079$, $\mathrm{cov}(k_1, k_3) = 0.724$, $\mathrm{cov}(k_2, k_3) = 0.569$, $\mathrm{cov}(k_1, c_1) = -0.183$, $\mathrm{cov}(k_1, c_2) = -0.087$, $\mathrm{cov}(k_2, c_1) = -0.363$, $\mathrm{cov}(k_2, c_2) = -0.531$, $\mathrm{cov}(k_3, c_1) = -0.279$, $\mathrm{cov}(k_3, c_2) = -0.316$, and $\mathrm{cov}(c_1, c_2) = 0.315$.

Supplementary Table S1. Kinetic data for PNA self-replication involving two different imidazole buffers at $T = 0$ °C.

Buffer	Model	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
1-MeIm	A	1.16	$(2.51\pm0.05)\times10^{-4}$	–	$(2.3\pm0.2)\times10^{-6}$	–
	B	1.21	$(2.64\pm0.07)\times10^{-4}$	$(0.3\pm1.7)\times10^{-4}$	$(2.8\pm0.2)\times10^{-6}$	0.1±0.6
Im	A	1.51	$(3.8\pm0.3)\times10^{-4}$	–	$(8\pm2)\times10^{-6}$	–
	B	0.896	$(3.1\pm0.2)\times10^{-4}$	$(3.3\pm0.4)\times10^{-3}$	$(1.1\pm0.1)\times10^{-5}$	11±2
5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.4 M buffer pH 7.5, 0 °C, 25 µl scale						

Supplementary Table S2. Optimized initial template concentrations for PNA self-replication involving two different imidazole buffers at $T = 0$ °C.

Buffer	model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)	c_2(exp.) (mM)	c_2(opt.) (mM)	Δc_2 (%)
1-MeIm	A	1.0	1.01±0.01	1	2.0	1.99±0.01	0.5
	B		1.02±0.01	2		1.98±0.01	1
Im	A	1.5	1.56±0.02	4	2.5	2.55±0.02	2
	B		1.54±0.01	3		2.50±0.01	0
5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.4 M buffer pH 7.5, 0 °C, 25 µl scale							



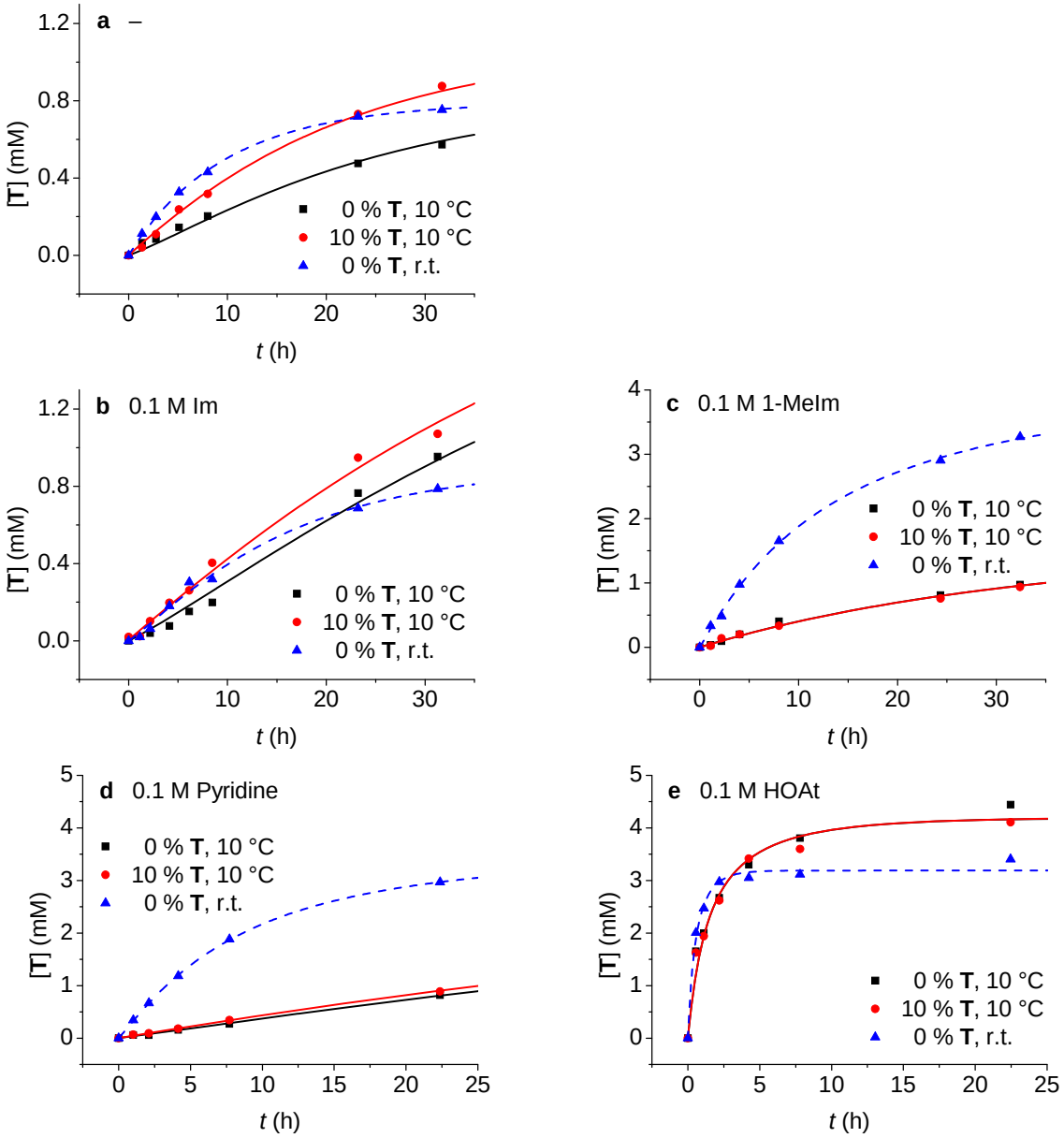
Supplementary Figure S6. Kinetics of PNA self-replication involving two different imidazole buffers at $T = 10$ °C (model B). Conditions: 5 mM tri-PNAs **A** and **B,** 0.2 M EDC, a: 0.4 M 1-methylimidazole pH 7.5, b: 0.4 M imidazole pH 7.5, 10 °C, 25 µl scale. a: $\mathrm{cov}(k_1, k_2) = -0.651$, $\mathrm{cov}(k_1, k_3) = 0.266$, $\mathrm{cov}(k_2, k_3) = 0.506$, $\mathrm{cov}(k_1, c_1) = -0.093$, $\mathrm{cov}(k_1, c_2) = 0.139$, $\mathrm{cov}(k_2, c_1) = -0.219$, $\mathrm{cov}(k_2, c_2) = -0.506$, $\mathrm{cov}(k_3, c_1) = -0.256$, $\mathrm{cov}(k_3, c_2) = -0.364$, and $\mathrm{cov}(c_1, c_2) = 0.255$. b: $\mathrm{cov}(k_1, k_2) = -0.234$, $\mathrm{cov}(k_1, k_3) = 0.504$, $\mathrm{cov}(k_2, k_3) = 0.658$, $\mathrm{cov}(k_1, c_1) = -0.015$, $\mathrm{cov}(k_1, c_2) = 0.099$, $\mathrm{cov}(k_2, c_1) = -0.402$, $\mathrm{cov}(k_2, c_2) = -0.534$, $\mathrm{cov}(k_3, c_1) = -0.226$, $\mathrm{cov}(k_3, c_2) = -0.259$, and $\mathrm{cov}(c_1, c_2) = 0.333$.

Supplementary Table S3. Kinetic Data for PNA self-replication involving two different imidazole buffers at $T = 10$ °C.

Buffer	Model	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
1-MeIm	A	1.22	$(6.3\pm0.1)\times10^{-4}$	–	$(0.6\pm2.7)\times10^{-7}$	–
	B	0.722	$(5.4\pm0.1)\times10^{-4}$	$(2.3\pm 0.3)\times10^{-3}$	$(0.08\pm1.73)\times10^{-7}$	4.3±0.6
Im	A	3.66	$(5.9\pm0.6)\times10^{-4}$	–	$(7\pm2)\times10^{-6}$	–
	B	1.25	$(3.6\pm0.2)\times10^{-4}$	$(9.0\pm0.6)\times10^{-3}$	$(9.2\pm0.6)\times10^{-6}$	25±3
5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.4 M buffer pH 7.5, 10 °C, 25 µl scale						

Supplementary Table S4. Optimized initial template concentrations for PNA self-replication involving two different imidazole buffers at $T = 0$ °C.

Buffer	model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)	c_2(exp.) (mM)	c_2(opt.) (mM)	Δc_2 (%)
1-MeIm	A	1.0	1.03±0.01	3	2.0	2.04±0.02	2
	B		1.02±0.01	2		2.01±0.01	1
Im	A	1.5	1.63±0.04	7	2.5	2.65±0.04	6
	B		1.55±0.02	3		2.50±0.02	0
5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.4 M buffer pH 7.5, 10 °C, 25 µl scale							



Supplementary Figure S7. Kinetics of PNA self-replication involving different nucleophilic catalysts (model B). Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.2, 0.2 M NaCl, b: 0.1 M imidazole, c: 0.1 M 1-methylimidazole, d: 0.1 M pyridine, e: 0.1 M HOAt, 6 µl scale. a/10 °C: $cov(k_1, k_2) = -0.393$, $cov(k_1, k_3) = 0.214$, $cov(k_2, k_3) = 0.778$, $cov(k_1, c_1) = 0.241$, $cov(k_2, c_1) = -0.683$, and $cov(k_3, c_1) = -0.458$; a/r.t.: $cov(k_1, k_2) = -0.980$, $cov(k_1, k_3) = -0.892$, and $cov(k_2, k_3) = 0.961$. b/10 °C: $cov(k_1, k_2) = -0.579$, $cov(k_1, k_3) = 0.000$, $cov(k_2, k_3) = 0.789$, $cov(k_1, c_1) = 0.265$, $cov(k_2, c_1) = -0.662$, and $cov(k_3, c_1) = -0.530$; b/r.t.: $cov(k_1, k_2) = -0.984$, $cov(k_1, k_3) = -0.932$, and $cov(k_2, k_3) = 0.980$. c/10 °C: $cov(k_1, k_2) = -0.531$, $cov(k_1, k_3) = 0.222$, $cov(k_2, k_3) = 0.737$, $cov(k_1, c_1) = 0.045$, $cov(k_2, c_1) = -0.630$, and $cov(k_3, c_1) = -0.608$; c/r.t.: $cov(k_1, k_2) = -0.974$, $cov(k_1, k_3) = -0.877$, and $cov(k_2, k_3) = 0.955$. d/10 °C: $cov(k_1, k_2) = -0.496$, $cov(k_1, k_3) = 0.241$, $cov(k_2, k_3) = 0.693$, $cov(k_1, c_1) = 0.059$, $cov(k_2, c_1) = -0.564$, and $cov(k_3, c_1) = -0.497$; d/r.t.: $cov(k_1, k_2) = -0.976$, $cov(k_1, k_3) = -0.830$, and $cov(k_2, k_3) = 0.916$. e/10 °C: $cov(k_1, k_2) = -0.967$, $cov(k_1, k_3) = -0.585$, $cov(k_2, k_3) = 0.712$, $cov(k_1, c_1) = 0.086$, $cov(k_2, c_1) = -0.153$, and $cov(k_3, c_1) = -0.063$; e/r.t.: $cov(k_1, k_2) = -0.987$, $cov(k_1, k_3) = -0.739$, and $cov(k_2, k_3) = 0.828$.

Supplementary Table S5. Kinetic data for PNA self-replication involving different nucleophilic catalysts.

Catalyst	T	Model	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
-	10 °C	A	2.92	$(3.7\pm0.5)\times10^{-4}$	–	$(4\pm3)\times10^{-6}$	–
		B	0.826	$(1.8\pm0.1)\times10^{-4}$	$(1.5\pm0.1)\times10^{-2}$	$(1.04\pm0.08)\times10^{-5}$	$(8\pm1)\times10^{1}$
	r.t.	A	0.257	$(9.17\pm0.09)\times10^{-4}$	–	$(2.22\pm0.03)\times10^{-5}$	–
		B	0.255	$(9.2\pm0.5)\times10^{-4}$	$(0.05\pm4.38)\times10^{-3}$	$(2.2\pm0.1)\times10^{-5}$	0.06±5.40
Im	10 °C	A	2.55	$(4.5\pm0.3)\times10^{-4}$	–	$(0.01\pm1.42)\times10^{-6}$	–
		B	1.46	$(2.6\pm0.2)\times10^{-4}$	$(1.0\pm0.1)\times10^{-2}$	$(2\pm1)\times10^{-6}$	38±3
	r.t.	A	1.09	$(5.4\pm0.2)\times10^{-4}$	–	$(9\pm1)\times10^{-6}$	–
		B	1.01	$(4\pm1)\times10^{-4}$	$(2\pm1)\times10^{-3}$	$(1.5\pm0.4)\times10^{-5}$	5±4
1-MeIm	10 °C	A	1.14	$(5.7\pm0.2)\times10^{-4}$	–	$(6.1\pm0.8)\times10^{-6}$	–
		B	1.24	$(5.3\pm0.3)\times10^{-4}$	$(0.1\pm1.6)\times10^{-3}$	$(4\pm1)\times10^{-6}$	0.2±3.2
	r.t.	A	1.82	$(3.29\pm0.07)\times10^{-3}$	–	$(3\pm4)\times10^{-7}$	–
		B	1.72	$(2.9\pm0.3)\times10^{-3}$	$(2\pm1)\times10^{-2}$	$(2\pm1)\times10^{-6}$	7±4
Pyridine	10 °C	A	1.19	$(5.0\pm0.2)\times10^{-4}$	–	$(0.2\pm1.2)\times10^{-6}$	–
		B	0.606	$(3.8\pm0.1)\times10^{-4}$	$(5.8\pm0.7)\times10^{-3}$	$(4\pm8)\times10^{-7}$	15±2
	r.t.	A	1.30	$(4.36\pm0.06)\times10^{-3}$	–	$(3.2\pm0.4)\times10^{-6}$	–
		B	0.448	$(3.3\pm0.1)\times10^{-3}$	$(5.0\pm0.5)\times10^{-2}$	$(7.2\pm0.4)\times10^{-6}$	15±2
HOAt	10 °C	A	7.81	$(3.4\pm0.2)\times10^{-2}$	–	$(1.7\pm0.3)\times10^{-5}$	–
		B	7.94	$(3.3\pm0.6)\times10^{-2}$	$(0.03\pm1.61)\times10^{-1}$	$(1.5\pm0.4)\times10^{-5}$	0.09±4.50
	r.t.	A	4.58	$(7.1\pm0.4)\times10^{-2}$	–	$(1.3\pm0.1)\times10^{-4}$	–
		B	4.62	$(7\pm2)\times10^{-2}$	$(0.06\pm7.22)\times10^{-1}$	$(1.2\pm0.2)\times10^{-4}$	0.09±10.83

5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.2, 0.2 M NaCl, 0.1 M catalyst, 6 µl scale

Supplementary Table S6. Optimized initial template concentrations for PNA self-replication involving different nucleophilic catalysts at T = 10 °C.

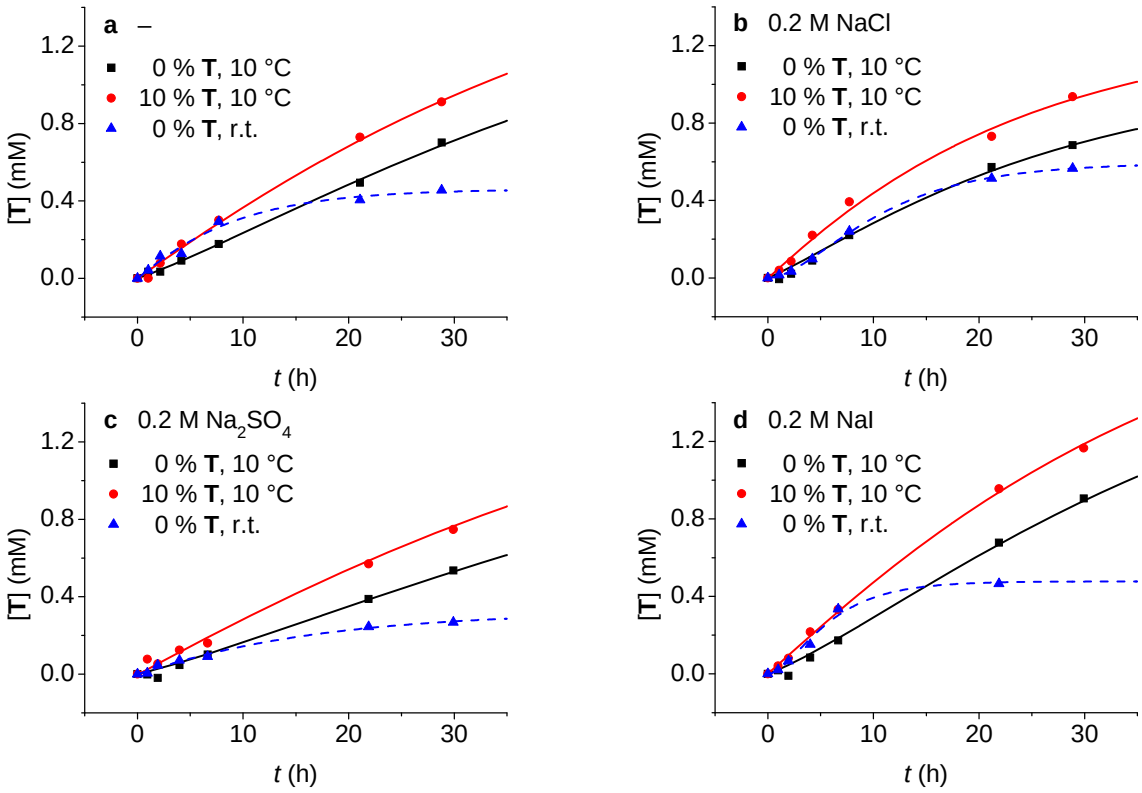
Catalyst	Model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)
–	A	0.50	0.57±0.03	14
	B		0.491±0.009	2
Im	A	0.50	0.54±0.02	8
	B		0.48±0.01	4
1-MeIm	A	0.50	0.47±0.01	6
	B		0.48±0.01	4
Pyridine	A	0.50	0.53±0.01	6
	B		0.511±0.07	2
HOAt	A	0.50	0.44±0.05	12
	B		0.44±0.05	12

5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.2, 0.2 M NaCl, 0.1 M catalyst, 10 °C, 6 µl scale

Supplementary Table S7. Product distribution without initially added template **T** in the presence or absence of different nucleophilic catalysts after 24 h reaction time.

Entry	Catalyst	*T* (°C)	**T**(%)	**A** (%)	**A+EDC** (%)	**S1*** (%)
1	–	10	14	46	30	10
2		r.t.	18	7	55	20
3	Im	10	24	73	1	2
4		r.t.	18	52	23	7
5	1-MeIm	10	28	69	1	2
6		r.t.	69	11	14	5
7	Pyridine	10	16	84	–	–
8		r.t.	61	28	4	7
9	HOAt	10	89	4	7	–
10		r.t.	62	25	13	–

5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.2, 0.2 M NaCl, 0.1 M catalyst, 6 µl scale
* a side product whose structure has not been determined yet, M = 1200 (MALDI-TOF), based on the retention time one can assume that it contains the fluoroaromatic isostere



Supplementary Figure S8. Kinetics of PNA self-replication involving different salts (model B). Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, a: no additional salt, b: 0.2 M NaCl, c: 0.2 M Na_2SO_4, d: 0.2 M NaI, 0.1 M imidazole, 6 µl scale. a/10 °C: cov(k_1, k_2) = -0.345, cov(k_1, k_3) = 0.256, cov(k_2, k_3) = 0.791, cov(k_1, c_1) = 0.186, cov(k_2, c_1) = -0.653, and cov(k_3, c_1) = -0.463; a/r.t.: cov(k_1, k_2) = -0.986, cov(k_1, k_3) = -0.925, and cov(k_2, k_3) = 0.974. b/10 °C: cov(k_1, k_2) = -0.429, cov(k_1, k_3) = 0.159, cov(k_2, k_3) = 0.793, cov(k_1, c_1) = 0.251, cov(k_2, c_1) = -0.681, and cov(k_3, c_1) = -0.483; b/r.t.: cov(k_1, k_2) = -0.996, cov(k_1, k_3) = -0.981, and cov(k_2, k_3) = -0.994. c/10 °C: cov(k_1, k_2) = -0.115, cov(k_1, k_3) = 0.418, cov(k_2, k_3) = 0.828, cov(k_1, c_1) = 0.084, cov(k_2, c_1) = -0.632, and cov(k_3, c_1) = -0.445; c/r.t.: cov(k_1, k_2) = -0.968, cov(k_1, k_3) = 0.874, and cov(k_2, k_3) = 0.966. d/10 °C: cov(k_1, k_2) = -0.480, cov(k_1, k_3) = 0.042, cov(k_2, k_3) = 0.834, cov(k_1, c_1) = 0.316, cov(k_2, c_1) = -0.684, and cov(k_3, c_1) = -0.504; d/r.t.: cov(k_1, k_2) = -0.978, cov(k_1, k_3) = 0.840, and cov(k_2, k_3) = 0.929.

Supplementary Table S8. Kinetic data for PNA self-replication involving different salts.

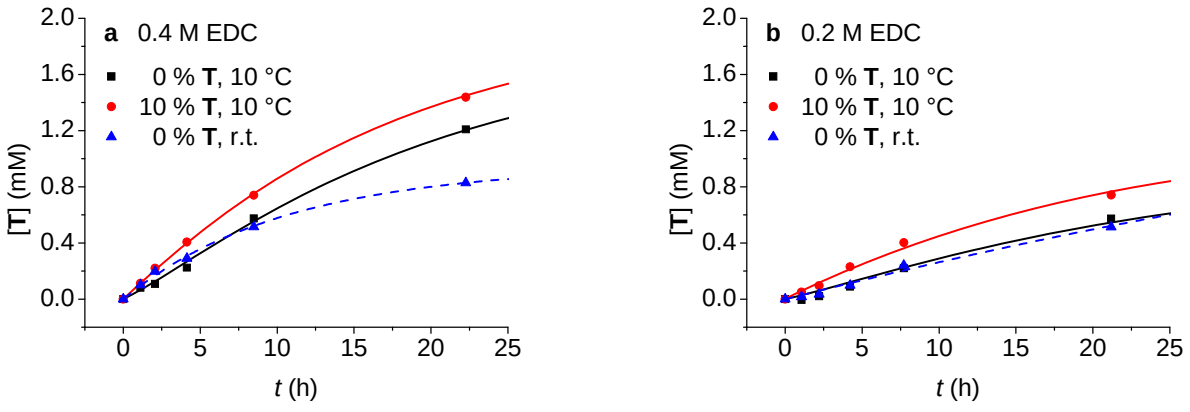
Salt	T	Model	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
–	10 °C	A	2.50	$(3.6\pm0.4)\times10^{-4}$	–	$(0.1\pm2.2)\times10^{-6}$	–
		B	0.650	$(1.83\pm0.08)\times10^{-4}$	$(1.10\pm0.06)\times10^{-2}$	$(3.3\pm0.6)\times10^{-6}$	60±5
	r.t.	A	0.941	$(5.6\pm0.3)\times10^{-4}$	–	$(2.5\pm0.2)\times10^{-5}$	–
		B	0.930	$(4\pm2)\times10^{-4}$	$(3\pm1)\times10^{-3}$	$(1\pm3)\times10^{-5}$	2±2
NaCl	10 °C	A	2.73	$(4.1\pm0.4)\times10^{-4}$	–	$(2\pm2)\times10^{-6}$	–
		B	0.993	$(2.3\pm0.1)\times10^{-4}$	$(1.5\pm0.1)\times10^{-2}$	$(9\pm1)\times10^{-6}$	52±6
	r.t.	A	1.07	$(3.7\pm0.2)\times10^{-4}$	–	$(7\pm2)\times10^{-6}$	–
		B	0.193	$(0.3\pm2.1)\times10^{-5}$	$(6.4\pm0.7)\times10^{-2}$	$(2.8\pm0.2)\times10^{-5}$	$(0.2\pm1.5)\times10^{5}$
Na_2SO_4	10 °C	A	2.65	$(2.7\pm0.4)\times10^{-4}$	–	$(0.3\pm3.1)\times10^{-6}$	–
		B	1.04	$(1.3\pm0.1)\times10^{-4}$	$(9\pm1)\times10^{-3}$	$(3\pm1)\times10^{-6}$	$(7\pm1)\times10^{1}$
	r.t.	A	0.388	$(2.0\pm0.1)\times10^{-4}$	–	$(1.3\pm0.1)\times10^{-5}$	–
		B	0.384	$(1.8\pm0.4)\times10^{-4}$	$(5\pm8)\times10^{-3}$	$(1.6\pm0.5)\times10^{-5}$	$(3\pm5)\times10^{1}$
NaI	10 °C	A	3.28	$(4.7\pm0.5)\times10^{-4}$	–	$(0.2\pm2.2)\times10^{-6}$	–
		B	0.821	$(2.0\pm0.1)\times10^{-4}$	$(1.57\pm0.09)\times10^{-2}$	$(3.6\pm0.6)\times10^{-6}$	79±9
	r.t.	A	1.56	$(6.5\pm0.6)\times10^{-4}$	–	$(2.6\pm0.4)\times10^{-5}$	–
		B	0.792	$(0.5\pm6.2)\times10^{-5}$	$(1\pm2)\times10^{-3}$	$(5.5\pm0.5)\times10^{-5}$	$(0.2\pm2.9)\times10^{3}$

5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M salt, 0.1 M imidazole, 6 µl scale

Supplementary Table S9. Optimized initial template concentrations for PNA self-replication involving different salts at T = 10 °C.

Salt	Model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)
–	A	0.50	0.55±0.02	10
	B		0.489±0.007	2
NaCl	A	0.50	0.57±0.02	14
	B		0.50±0.01	0
Na_2SO_4	A	0.50	0.58±0.02	16
	B		0.53±0.01	6
NaI	A	0.50	0.56±0.03	12
	B		0.493±0.009	1

5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M salt, 0.1 M imidazole, 10 °C, 6 µl scale



Supplementary Figure S9. Effect of EDC concentration on the kinetics of PNA self-replication (model B). Conditions: 5 mM tri-PNAs **A** and **B**, a: 0.4 M EDC, b: 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 6 µl scale. a/10 °C: $\mathrm{cov}(k_1, k_2) = -0.760$, $\mathrm{cov}(k_1, k_3) = -0.264$, $\mathrm{cov}(k_2, k_3) = 0.797$, $\mathrm{cov}(k_1, c_1) = 0.411$, $\mathrm{cov}(k_2, c_1) = -0.687$, and $\mathrm{cov}(k_3, c_1) = -0.548$; a/r.t.: $\mathrm{cov}(k_1, k_2) = -0.982$, $\mathrm{cov}(k_1, k_3) = -0.910$, and $\mathrm{cov}(k_2, k_3) = 0.970$. b/10 °C: $\mathrm{cov}(k_1, k_2) = -0.484$, $\mathrm{cov}(k_1, k_3) = 0.101$, $\mathrm{cov}(k_2, k_3) = 0.780$, $\mathrm{cov}(k_1, c_1) = 0.231$, $\mathrm{cov}(k_2, c_1) = -0.680$, and $\mathrm{cov}(k_3, c_1) = -0.503$; b/r.t.: $\mathrm{cov}(k_1, k_2) = 1.161$, $\mathrm{cov}(k_1, k_3) = 1.391$, and $\mathrm{cov}(k_2, k_3) = -1.048$.

Supplementary Table S10. Kinetic data for PNA self-replication involving different EDC concentrations.

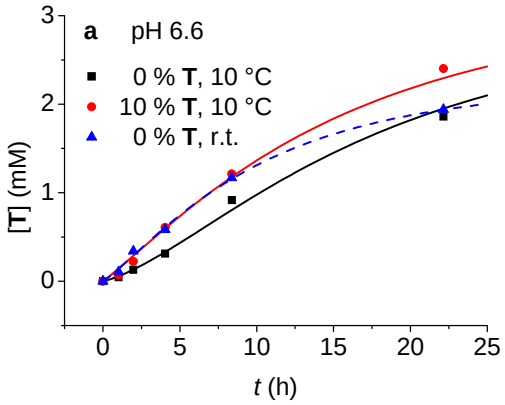
[EDC] (M)	T	Model	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
0.4	10 °C	A	2.79	$(9.2\pm0.6)\times10^{-4}$	–	$(1\pm2)\times10^{-6}$	–
		B	0.667	$(5.2\pm0.2)\times10^{-4}$	$(2.7\pm0.1)\times10^{-2}$	$(8.8\pm0.5)\times10^{-6}$	52±4
	r.t.	A	0.602	$(1.02\pm0.02)\times10^{-3}$	–	$(1.93\pm0.09)\times10^{-5}$	–
		B	0.622	$(1.0\pm0.1)\times10^{-3}$	$(0.3\pm1.3)\times10^{-2}$	$(2.0\pm0.4)\times10^{-5}$	3±13
0.2	10 °C	A	2.31	$(4.0\pm0.5)\times10^{-4}$	–	$(1\pm3)\times10^{-6}$	–
		B	1.05	$(2.4\pm0.2)\times10^{-4}$	$(1.6\pm0.2)\times10^{-2}$	$(1.0\pm0.2)\times10^{-5}$	$(7\pm1)\times10^{1}$
	r.t.	A	0.888	$(3.2\pm0.2)\times10^{-4}$	–	$(2\pm2)\times10^{-6}$	–
		B	0.811	$(2.9\pm0.4)\times10^{-4}$	$(3\pm9)\times10^{-3}$	$(2\pm6)\times10^{-6}$	$(1\pm3)\times10^{1}$

5 mM tri-PNAs **A** and **B**, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, 6 µl scale

Supplementary Table S11. Optimized initial template additions for PNA self-replication involving different EDC concentrations at T = 10 °C.

[EDC] (M)	Model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)
0.4	A	0.50	0.59±0.03	18
	B		0.506±0.008	2
0.2	A	0.50	0.56±0.02	12
	B		0.49±0.01	2

5 mM tri-PNAs **A** and **B**, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, 10 °C, 6 µl scale



Supplementary Figure S10. Kinetics of PNA self-replication at pH 6.6 (model B). Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS, 0.2 M NaCl, 0.1 M imidazole, 6 µl scale. 10 °C: cov(k_1, k_2) = -0.860, cov(k_1, k_3) = -0.540, cov(k_2, k_3) = 0.859, cov(k_1, c_1) = 0.553, cov(k_2, c_1) = -0.725, and cov(k_3, c_1) = -0.590; r.t.: cov(k_1, k_2) = -0.974, cov(k_1, k_3) = -0.848, and cov(k_2, k_3) = 0.937.

Supplementary Table S12. Kinetic data for PNA self-replication under different pH conditions.

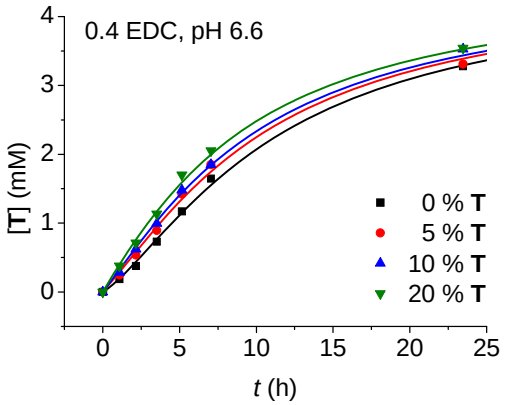
pH	T	Model	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
6.6	10 °C	A	6.74	$(1.8\pm0.2)\times10^{-4}$	–	$(0.6\pm2.7)\times10^{-6}$	–
		B	2.79	$(3.5\pm0.8)\times10^{-4}$	$(6.0\pm0.7)\times10^{-2}$	$(5\pm1)\times10^{-6}$	$(1.7\pm0.6)\times10^{2}$
	r.t.	A	1.95	$(2.06\pm0.07)\times10^{-3}$	–	$(5\pm1)\times10^{-6}$	–
		B	1.24	$(1.2\pm0.2)\times10^{-3}$	$(6\pm1)\times10^{-2}$	$(1.4\pm0.2)\times10^{-5}$	$(5\pm2)\times10^{1}$
7.2	10 °C	A	2.55	$(4.5\pm0.3)\times10^{-4}$	–	$(0.01\pm1.42)\times10^{-6}$	–
		B	1.46	$(2.6\pm0.2)\times10^{-4}$	$(1.0\pm0.1)\times10^{-2}$	$(2\pm1)\times10^{-6}$	38±3
	r.t.	A	1.09	$(5.4\pm0.2)\times10^{-4}$	–	$(9\pm1)\times10^{-6}$	–
		B	1.01	$(4\pm1)\times10^{-4}$	$(2\pm1)\times10^{-3}$	$(1.5\pm0.4)\times10^{-5}$	5±4
7.6	10 °C	A	2.73	$(4.1\pm0.4)\times10^{-4}$	–	$(2\pm2)\times10^{-6}$	–
		B	0.993	$(2.3\pm0.1)\times10^{-4}$	$(1.5\pm0.1)\times10^{-2}$	$(9\pm1)\times10^{-6}$	52±6
	r.t.	A	1.07	$(3.7\pm0.2)\times10^{-4}$	–	$(7\pm2)\times10^{-6}$	–
		B	0.193	$(0.3\pm2.1)\times10^{-5}$	$(6.4\pm0.7)\times10^{-2}$	$(2.8\pm0.2)\times10^{-5}$	$(0.2\pm1.5)\times10^{5}$

5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS, 0.2 M NaCl, 0.1 M imidazole, 6 µl scale

Supplementary Table S13. Optimized initial template additions for PNA self-replication under different pH conditions at T = 10 °C.

pH	Model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)
6.6	A	0.50	0.60±0.06	20
	B		0.44±0.03	12
7.2	A	0.50	0.54±0.02	8
	B		0.48±0.01	4
7.6	A	0.50	0.57±0.02	14
	B		0.50±0.01	0

5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS, 0.2 M NaCl, 0.1 M imidazole, 6 µl scale



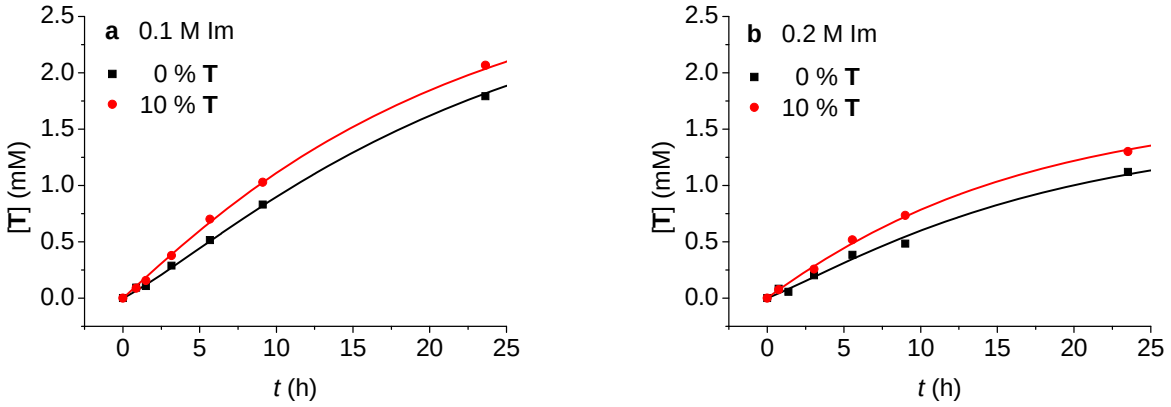
Supplementary Figure S11. Kinetics of PNA self-replication under conditions optimized for fast replication and high conversion (model B). Conditions: 5 mM tri-PNAs **A** and **B**, 0.4 M EDC, 0.2 M MOPS pH 6.6, 0.1 M imidazole, 10 °C, 6 µl scale. cov(k_1, k_2) = -0.928, cov(k_1, k_3) = -0.582, cov(k_2, k_3) = 0.756, cov(k_1, c_1) = 0.046, cov(k_1, c_2) = 0.277, cov(k_1, c_3) = 0.475, cov(k_2, c_1) = -0.218, cov(k_2, c_2) = -0.433, cov(k_2, c_3) = -0.609, cov(k_3, c_1) = -0.168, cov(k_3, c_2) = -0.312, cov(k_3, c_3) = -0.433, cov(c_1, c_2) = 0.285, cov(c_1, c_3) = 0.278, and cov(c_2, c_3) = 0.388.

Supplementary Table S14. Kinetic Data for PNA self-replication under conditions optimized for fast replication and high conversion.

Model	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
A	6.30	$(4.2\pm0.1)\times10^{-3}$	–	$(0.7\pm9.5)\times10^{-7}$	–
B	2.34	$(1.3\pm0.1)\times10^{-3}$	$(9.4\pm0.5)\times10^{-2}$	$(2.1\pm0.4)\times10^{-7}$	72±9
5 mM tri-PNAs **A** and **B**, 0.4 M EDC, 0.2 M MOPS pH 6.6, 0.1 M imidazole, 10 °C, 6 µl					

Supplementary Table S15. Optimized initial template concentrations for PNA self-replication under conditions optimized for fast replication and high conversion.

Model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)	c_2(exp.) (mM)	c_2(opt.) (mM)	Δc_2 (%)	c_3(exp.) (mM)	c_3(opt.) (mM)	Δc_3 (%)
A	0.25	0.28±0.03	12	0.50	0.53±0.06	6	1.0	1.09±0.06	9
B		0.25±0.02	0		0.47±0.02	6		0.90±0.02	10
5 mM tri-PNAs **A** and **B**, 0.4 M EDC, 0.2 M MOPS pH 6.6, 0.1 M imidazole, 10 °C, 6 µl									



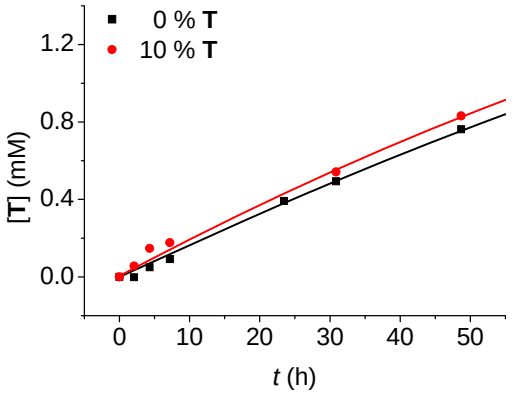
Supplementary Figure S12. Kinetics of PNA self-replication involving different imidazole concentrations (model B). Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.2, 0.2 M NaI, a: 0.1 M imidazole, b: 0.2 M imidazole, 10 °C, 6 µl scale. a: $\mathrm{cov}(k_1, k_2) = -0.864$, $\mathrm{cov}(k_1, k_3) = -0.499$, $\mathrm{cov}(k_2, k_3) = 0.837$, $\mathrm{cov}(k_1, c_1) = 0.447$, $\mathrm{cov}(k_2, c_1) = -0.654$, and $\mathrm{cov}(k_3, c_1) = -0.570$. b: $\mathrm{cov}(k_1, k_2) = -0.767$, $\mathrm{cov}(k_1, k_3) = -0.218$, and $\mathrm{cov}(k_2, k_3) = 0.753$, $\mathrm{cov}(k_1, c_1) = 0.404$, $\mathrm{cov}(k_2, c_1) = -0.677$, and $\mathrm{cov}(k_3, c_1) = -0.509$.

Supplementary Table S16. Kinetic data for PNA self-replication under different imidazole concentrations.

[Im] (mM)	Model	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
0.1	A	3.25	$(1.42\pm0.07)\times10^{-3}$	–	$(0.1\pm1.2)\times10^{-6}$	–
	B	0.850	$(6.9\pm0.3)\times10^{-4}$	$(3.1\pm0.2)\times10^{-2}$	$(3.5\pm0.4)\times10^{-6}$	45±5
0.2	A	2.63	$(9.0\pm0.5)\times10^{-4}$	–	$(4\pm2)\times10^{-6}$	–
	B	1.16	$(5.4\pm0.3)\times10^{-4}$	$(2.4\pm0.2)\times10^{-2}$	$(1.10\pm0.09)\times10^{-5}$	44±6
5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.2, 0.2 M NaI, 10 °C, 6 µl scale						

Supplementary Table S17. Optimized initial template concentrations for PNA self-replication under different imidazole concentrations at T = 10 °C.

[Im] (M)	Model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)
0.1	A	0.50	0.56±0.03	12
	B		0.483±0.009	3
0.2	A	0.50	0.58±0.03	16
	B		0.50±0.01	0
5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.2, 0.2 M NaI, 10 °C, 6 µl scale				



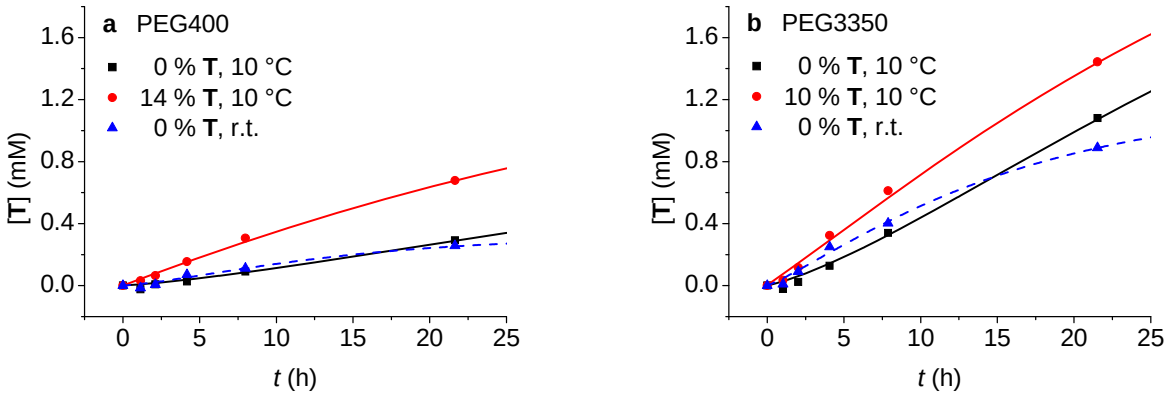
Supplementary Figure S13. Kinetics of PNA self-replication in the eutectic phase (model B). Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, -19 °C, 12 µl scale. cov(k_1, k_2) = -0.398, cov(k_1, k_3) = 0.382, cov(k_2, k_3) = 0.669, cov(k_1, c_1) = -0.015, cov(k_2, c_1) = -0.402, and cov(k_3, c_1) = -0.226.

Supplementary Table S18. Kinetic data for PNA self-replication in the eutectic phase at T = -19 °C and in the liquid phase at T = 10 °C.

T	Model	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
-19 °C	A	1.24	$(2.1\pm0.1)\times10^{-4}$	–	$(1\pm7)\times10^{-7}$	–
	B	0.978	$(1.70\pm0.09)\times10^{-4}$	$(1.8\pm0.5)\times10^{-3}$	$(3\pm6)\times10^{-7}$	11±4
10 °C	A	2.73	$(4.1\pm0.4)\times10^{-4}$	–	$(2\pm2)\times10^{-6}$	–
	B	0.993	$(2.3\pm0.1)\times10^{-4}$	$(1.5\pm0.1)\times10^{-2}$	$(9\pm1)\times10^{-6}$	52±6
5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, -19 °C, 12 µl scale						

Supplementary Table S19. Optimized initial template concentrations for PNA self-replication in the eutectic phase at T = -19 °C.

Model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)
A	0.50	0.54±0.01	8
B		0.52±0.01	4
5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, -19 °C, 12 µl scale			



Supplementary Figure S14. Effect of PEG on the kinetics of PNA self-replication (model B). Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, a: 20 % PEG400, b: 20 % PEG3350, 6 µl scale. a/10 °C: cov(k_1, k_2) = -0.179, cov(k_1, k_3) = 0.205, cov(k_2, k_3) = 0.885, cov(k_1, c_1) = 0.157, cov(k_2, c_1) = -0.653, and cov(k_3, c_1) = -0.468; a/r.t.: cov(k_1, k_2) = -0.936, cov(k_1, k_3) = -0.764, and cov(k_2, k_3) = 0.937. b/10 °C: cov(k_1, k_2) = -0.675, cov(k_1, k_3) = -0.277, cov(k_2, k_3) = 0.873, cov(k_1, c_1) = 0.448, cov(k_2, c_1) = -0.735, and cov(k_3, c_1) = -0.602; b/r.t.: cov(k_1, k_2) = -0.981, cov(k_1, k_3) = -0.924, and cov(k_2, k_3) = 0.978.

Supplementary Table S20. Kinetic data for PNA self-replication in the absence and presence of PEG.

PEG	T	Fit	RMS (%)	k_1 (M^{-1} s^{-1})	k_2 ($M^{-3/2}$ s^{-1})	k_3 (s^{-1})	ε ($M^{-1/2}$)
–	10 °C	A	2.31	$(4.0\pm0.5)\times10^{-4}$	–	$(1\pm3)\times10^{-6}$	–
		B	1.05	$(2.4\pm0.2)\times10^{-4}$	$(1.6\pm0.2)\times10^{-2}$	$(1.0\pm0.2)\times10^{-5}$	$(7\pm1)\times10^{1}$
	r.t.	A	0.888	$(3.2\pm0.2)\times10^{-4}$	–	$(2\pm2)\times10^{-6}$	–
		B	0.811	$(2.9\pm0.4)\times10^{-4}$	$(3\pm9)\times10^{-3}$	$(2\pm6)\times10^{-6}$	$(1\pm3)\times10^{1}$
400	10 °C	A	3.54	$(2.6\pm0.7)\times10^{-4}$	–	$(0.5\pm7.3)\times10^{-6}$	–
		B	0.592	$(5.3\pm0.5)\times10^{-5}$	$(1.37\pm0.07)\times10^{-2}$	$(5\pm1)\times10^{-6}$	$(2.6\pm0.4)\times10^{2}$
	r.t.	A	0.757	$(1.5\pm0.2)\times10^{-4}$	–	$(2\pm4)\times10^{-6}$	–
		B	0.642	$(5\pm4)\times10^{-5}$	$(3\pm1)\times10^{-2}$	$(2.3\pm0.6)\times10^{-6}$	$(6\pm7)\times10^{2}$
3350	10 °C	A	4.58	$(7.8\pm0.4)\times10^{-4}$	–	$(1\pm2)\times10^{-11}$	–
		B	1.38	$(2.1\pm0.2)\times10^{-4}$	$(2.6\pm0.2)\times10^{-2}$	$(1\pm11)\times10^{-6}$	$(1.2\pm0.2)\times10^{2}$
	r.t.	A	1.10	$(6.5\pm0.3)\times10^{-4}$	–	$(4\pm1)\times10^{-6}$	–
		B	0.980	$(4\pm1)\times10^{-4}$	$(3\pm2)\times10^{-2}$	$(1.4\pm0.5)\times10^{-5}$	$(8\pm7)\times10^{1}$

5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, 20 % PEG, 6 µl scale

Supplementary Table S21. Optimized initial template concentrations for PNA self-replication in the absence and presence of PEG at T = 10 °C.

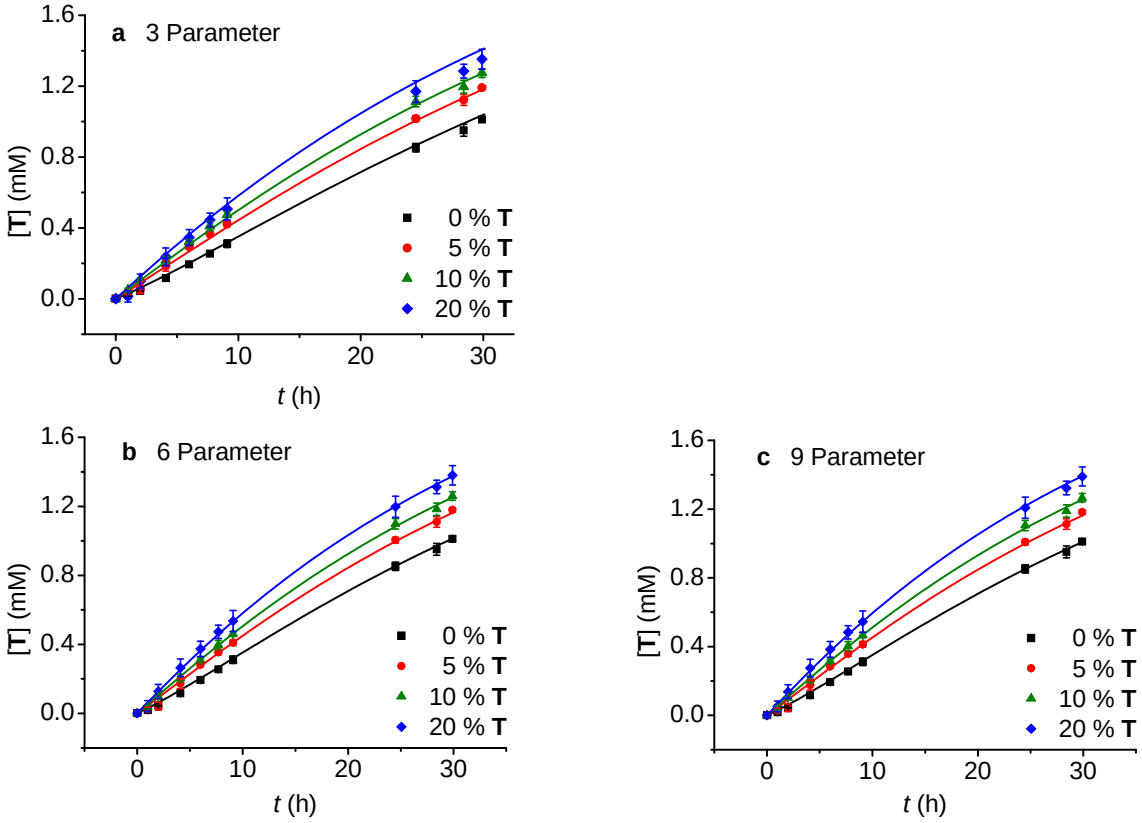
PEG	Model	c_1(exp.) (mM)	c_1(opt.) (mM)	Δc_1 (%)
400	A	0.7	0.78±0.04	11
	B		0.688±0.007	2
3350	A	0.5	0.54±0.05	8
	B		0.46±0.02	8

5 mM tri-PNAs **A** and **B**, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, 10 °C, 6 µl scale

Supplementary Table S22. Product distribution without initially added template **T** and in the presence or absence of PEG after 24 h reaction time.

Entry	*T* (°C)	PEG	**T** (%)	**A** (%)	**A+EDC** (%)	**S1*** (%)
1	10	–	12	83	2	3
2	r.t.		11	70	7	12
3	10	400	7	84	4	5
4	r.t.		6	62	10	22
5	10	3350	25	69	3	3
6	r.t.		19	45	11	25

5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, 20 % PEG, 6 µl scale
* a side product whose structure has not been determined yet, M = 1200 (MALDI-TOF), based on the retention time one can assume that it contains the fluoroaromatic isostere



Supplementary Figure S15. Fitting with (a) three (k_1-k_3), (b) six (k_1-k_3 and c_1-c_3), and (c) nine (k_1-k_3, c_1-c_3, and a_1-a_3) parameters according to equations (1)-(3). The approximated initial template concentrations were subtracted from the experimental values to enable comparison of template production. Reactions were carried out in the presence of the amount of template **T** shown in the legend using otherwise identical reaction conditions (5 mM **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, 10 °C, 16 µl scale). Error bars indicate the standard deviation due to experimental inaccuracies and HPLC integration over three independent series of experiments. The off-diagonal elements of the covariance matrix for the 6 parameter fitting were: cov(k_1, k_2) = -0.569, cov(k_1, k_3) = 0.043, cov(k_2, k_3) = 0.766, cov(k_1, c_1) = -0.138, cov(k_1, c_2) = 0.075, cov(k_1, c_3) = 0.276, cov(k_2, c_1) = -0.189, cov(k_2, c_2) = -0.411, cov(k_2, c_3) = -0.605, cov(k_3, c_1) = -0.232, cov(k_3, c_2) = -0.346, cov(k_3, c_3) = -0.439, cov(c_1, c_2) = 0.230, cov(c_1, c_3) = 0.223, and cov(c_2, c_3) = 0.344.

4 SimFit Files

4.1 Sample *SimFit* command file

```
* Figure 3
* 5mM A und B, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M Imidazol, 10 °C, 16 µl scale

* Define and scale observable data to be read from input data file serie123_averaged.txt; format of commands:
* DEFINE (running number, name of observable as in headline, type (e, p, i or c for educt, product, intermediate or *
* catalyst, respectively), screen color during plot); SCALE (number of scale function, calibration data):
define (1, t, p, 5) scale (3, 1)
define (2, a, e, 1) scale (3, 1)

* Read the input data file serie123_averaged.txt:
read (serie123_averaged)

* Select observable/s for screen plots:
select (t, a)

* Select experiment/s in the input data set:
choose (expall)

* Input of reaction equations and input of estimates for the rate constants of the above reactions. Rate constants may be
* variable, fixed or coupled. Format of commands: REACTION (expression) CONSTANT (number of reaction
* equation, numerical value of rate constant, number of variable (zero means fixed), coupling factor, minmax-factor):
reaction (   A + B  --> T)  constant (1, 6E-4 , 1, 1, 1000)
reaction (        A --> X)  constant (2, 1E-6 , 2, 1, 1000)
reaction (A + B + 0.5 T --> 1.5 T) constant (3, 1e-2, 3, 1, 1000)

* Encode rate equations:
reaction (compile)

* Print rate equations and Jacobian matrix onto the screen:
reaction (show)

* Print constants onto the screen:
constant (show)

* Input of assignments. ASSIGN (obs, X = Y) means: the theoretical concentration of the observable X is taken from
* the species Y. Symbolic expressions as ASSIGN (obs, X = A + B) are possible. ASSIGN (spec, X = Y) means: the
* initial concentration of species X is taken from the observable Y:
assign (obs , T = T)
assign (obs , A = A + X)
assign (spec, A = A)
assign (spec, B = #0.005)
assign (spec, T = t)

* Set up observables for optimization. Format of command: CZERO (number of parameter for iteration, name of the
* observable whose initial value needs optimization, number of the file in which the observable exists, number of the
* experiment within the file, minmax-factor):
czero (4, t, 1, 2, 1.2)
czero (5, t, 1, 3, 1.2)
czero (6, t, 1, 4, 1.2)

* Define units of time and concentration for screen plots:
time (h)
conc (mM)
```

```
* Define window in concentration-time space; format of command: WIN (min. time, max. time, time-axis interval,
* lenght of conc. tic, min. conc., max. conc., conc.-axis interval, lenght of time tic):
win (0,30,5,.3,0,5,1,.05)

* Specify number of variable rate constants and initial concentrations (which are treated as rate constants):
dim (6)

* Set the type of integration to Runge-Kutta-Nystrom:
int (rkn)

* Set the maximal number of iteration steps for Simplex and Newton:
numiter (1000)

* Sets the number of plotsteps
numplot (500)

* Show list of integration parameters:
int (show)

* Print species and residues onto the screen:
plot (spec, resi)

* Start the optimization using the Simplex algorithm two times (1000 cycles each) followed by the Newton-Raphson
* algorithm.
simplex (plot)
simplex (plot)
newton (plot)

* Determine the optimisation parameter. Parameter affects Newton optimisation, which behaves 'quick and dirty' when
* #optim is small and 'slow but accurate' when #optim is large:
opar (1e+5)

* Generate a plot of RMS as the function of iteration parameters. If only two parameters k1 and k2 were iterated, a
* single plot is generated showing RMS as a function of k1 and k2. If N parameters were iterated, N*(N-1)/2 plots will
* show up on the screen. The minimum is expected to be in the centre of each plot area. Format of command:
* SCAN (factor by which a parameter is multiplied in the next step, number of steps before or after the centre of each
* axis, Maximum of RMS):
scan (1.03, 25, 10)

* Plot to file. The filename is extended by the symbol p (observable Points) or c (theoretical Curves). If the respective
* data file contains one experiment, 1 will follow behind p or c. If N experiments are included in the data file, 2*N files
* will be generated having the extensions p1, p2, p3, c1, c2, c3:
plot (file)
```

4.2 Concentration-time data

4.2.1 Replication assays using 1-methylimidazole buffer

Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.4 M 1-methylimidazole pH 7.5, 25 µl scale.

T = 0 °C

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	4.25	0.000065	0.004935	0.000065
3	1	8.01	0.000166	0.004834	0.000166
4	1	22.72	0.000503	0.004497	0.000503
5	1	30.69	0.000537	0.004463	0.000537
6	1	47.77	0.000719	0.004281	0.000719
7	1	54.93	0.000786	0.004214	0.000786
8	1	71.18	0.000937	0.004063	0.000937
9	1	94.73	0.001168	0.003832	0.001168
1	2	0.00	0.000000	0.005000	0.001000
2	2	4.25	0.000085	0.004915	0.001085
3	2	8.01	0.000182	0.004818	0.001182
4	2	22.72	0.000447	0.004553	0.001447
5	2	30.69	0.000550	0.004450	0.001550
6	2	47.77	0.000771	0.004229	0.001771
7	2	54.93	0.000797	0.004203	0.001797
8	2	71.18	0.000964	0.004036	0.001964
9	2	94.73	0.001182	0.003818	0.002182
1	3	0.00	0.000000	0.005000	0.002000
2	3	4.25	0.000121	0.004879	0.002121
3	3	8.01	0.000189	0.004811	0.002189
4	3	22.72	0.000470	0.004530	0.002470
5	3	30.69	0.000515	0.004485	0.002515
6	3	47.77	0.000741	0.004259	0.002741
7	3	54.93	0.000823	0.004177	0.002823
8	3	71.18	0.000955	0.004045	0.002955
9	3	94.73	0.001029	0.003971	0.003029
end					

T = 10 °C

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	4.15	0.000201	0.004799	0.000201
3	1	8.06	0.000402	0.004598	0.000402
4	1	22.77	0.000940	0.004060	0.000940
5	1	47.77	0.001731	0.003269	0.001731
1	2	0.00	0.000000	0.005000	0.001000
2	2	4.15	0.000259	0.004741	0.001259
3	2	8.06	0.000437	0.004563	0.001437
4	2	22.77	0.001007	0.003993	0.002007
5	2	47.77	0.001792	0.003208	0.002792
1	3	0.00	0.000000	0.005000	0.002000
2	3	4.15	0.000262	0.004738	0.002262
3	3	8.06	0.000459	0.004541	0.002459
4	3	22.77	0.001049	0.003951	0.003049
5	3	47.77	0.001816	0.003184	0.003816
end					

4.2.2 Replication assays using imidazole buffer

Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.4 M imidazole pH 7.5, 25 µl scale.

T = 0 °C

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.00500	0.0000001
2	1	3.58	0.000097	0.00490	0.000097
3	1	6.58	0.000155	0.00485	0.000155
4	1	20.46	0.000385	0.00461	0.000385
5	1	29.77	0.000527	0.00447	0.000527
1	2	0.00	0.000000	0.00500	0.001500
2	2	3.58	0.000194	0.00481	0.001694
3	2	6.58	0.000227	0.00477	0.001727
4	2	20.46	0.000526	0.00447	0.002026
5	2	29.77	0.000673	0.00433	0.002173
1	3	0.00	0.000000	0.00500	0.002500
2	3	3.58	0.000182	0.00482	0.002682
3	3	6.58	0.000249	0.00475	0.002749
4	3	20.46	0.000509	0.00449	0.003009
5	3	29.77	0.000632	0.00437	0.003132
end					

T = 10 °C

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	2.33	0.000041	0.004959	0.000041
3	1	6.63	0.000198	0.004802	0.000198
4	1	20.52	0.000569	0.004431	0.000569
5	1	29.80	0.000723	0.004277	0.000723
6	1	44.65	0.000922	0.004078	0.000922
1	2	0.00	0.000000	0.005000	0.001500
2	2	2.33	0.000237	0.004763	0.001737
3	2	6.63	0.000454	0.004546	0.001954
4	2	20.52	0.000856	0.004144	0.002356
5	2	29.80	0.001032	0.003968	0.002532
6	2	44.65	0.001176	0.003824	0.002676
1	3	0.00	0.000000	0.005000	0.002500
2	3	2.33	0.000223	0.004777	0.002723
3	3	6.63	0.000401	0.004599	0.002901
4	3	20.52	0.000871	0.004129	0.003371
5	3	29.80	0.001085	0.003915	0.003585
6	3	44.65	0.001281	0.003719	0.003781
end					

4.2.3 Replication assays involving different nucleophilic catalysts

Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.2, 0.2 M NaCl, 6 µl scale, exp.1-2: T = 10 °C, exp.3: T = r.t.

a: no catalyst

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.35	0.000065	0.004935	0.000065
3	1	2.77	0.000084	0.004916	0.000084
4	1	5.09	0.000145	0.004855	0.000145
5	1	8.00	0.000203	0.004797	0.000203
6	1	23.22	0.000476	0.004524	0.000476
7	1	31.72	0.000572	0.004428	0.000572
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.35	0.000031	0.004969	0.000531
3	2	2.77	0.000099	0.004901	0.000599
4	2	5.09	0.000227	0.004773	0.000727
5	2	8.00	0.000308	0.004692	0.000808
6	2	23.22	0.000721	0.004279	0.001221
7	2	31.72	0.000866	0.004134	0.001366
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.35	0.000112	0.004888	0.000112
3	3	2.77	0.000199	0.004801	0.000199
4	3	5.09	0.000327	0.004673	0.000327
5	3	8.00	0.000431	0.004569	0.000431
6	3	23.22	0.000719	0.004281	0.000719
7	3	31.72	0.000754	0.004246	0.000754
end					

b: 0.1 M Imidazole

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	2.18	0.000040	0.004960	0.000040
3	1	4.13	0.000077	0.004923	0.000077
4	1	6.13	0.000152	0.004848	0.000152
5	1	8.47	0.000199	0.004801	0.000199
6	1	23.22	0.000765	0.004235	0.000765
7	1	31.28	0.000954	0.004046	0.000954
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.13	0.000003	0.004997	0.000503
3	2	2.18	0.000081	0.004919	0.000581
4	2	4.13	0.000177	0.004823	0.000677
5	2	6.13	0.000241	0.004759	0.000741
6	2	8.47	0.000384	0.004616	0.000884
7	2	23.22	0.000928	0.004072	0.001428
8	2	31.28	0.001052	0.003948	0.001552
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.13	0.000020	0.004980	0.000020
3	3	2.18	0.000062	0.004938	0.000062
4	3	4.13	0.000180	0.004820	0.000180
5	3	6.13	0.000304	0.004696	0.000304
6	3	8.47	0.000319	0.004681	0.000319
7	3	23.22	0.000687	0.004313	0.000687
8	3	31.28	0.000787	0.004213	0.000787
end					

c: 0.1 M 1-Methylimidazole

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.11	0.000038	0.004962	0.000038
3	1	2.21	0.000093	0.004907	0.000093
4	1	4.05	0.000201	0.004799	0.000201
5	1	8.02	0.000404	0.004596	0.000404
6	1	24.35	0.000810	0.004190	0.000810
7	1	32.40	0.000975	0.004025	0.000975
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.11	0.000005	0.004995	0.000505
3	2	2.21	0.000120	0.004880	0.000620
4	2	4.05	0.000182	0.004818	0.000682
5	2	8.02	0.000314	0.004686	0.000814
6	2	24.35	0.000738	0.004262	0.001238
7	2	32.40	0.000919	0.004081	0.001419
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.11	0.000335	0.004665	0.000335
3	3	2.21	0.000485	0.004515	0.000485
4	3	4.05	0.000975	0.004025	0.000975
5	3	8.02	0.001651	0.003349	0.001651
6	3	24.35	0.002905	0.002095	0.002905
7	3	32.40	0.003270	0.001730	0.003270
end					

d: 0.1 M Pyridine

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.02	0.000062	0.004938	0.000062
3	1	2.10	0.000056	0.004944	0.000056
4	1	4.15	0.000156	0.004844	0.000156
5	1	7.72	0.000270	0.004730	0.000270
6	1	22.37	0.000820	0.004180	0.000820
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.02	0.000078	0.004922	0.000578
3	2	2.10	0.000102	0.004898	0.000602
4	2	4.15	0.000194	0.004806	0.000694
5	2	7.72	0.000356	0.004644	0.000856
6	2	22.37	0.000900	0.004100	0.001400
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.02	0.000343	0.004657	0.000343
3	3	2.10	0.000669	0.004331	0.000669
4	3	4.15	0.001184	0.003816	0.001184
5	3	7.72	0.001882	0.003118	0.001882
6	3	22.37	0.002968	0.002032	0.002968
end					

e: 0.1 M HOAt

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	0.56	0.001652	0.003348	0.001652
3	1	1.11	0.001996	0.003004	0.001996
4	1	2.19	0.002671	0.002329	0.002671
5	1	4.24	0.003300	0.001700	0.003300
6	1	7.81	0.003805	0.001195	0.003805
7	1	22.46	0.004443	0.000557	0.004443
1	2	0.00	0.000000	0.005000	0.000500
2	2	0.56	0.001567	0.003433	0.002067
3	2	1.11	0.001881	0.003119	0.002381
4	2	2.19	0.002560	0.002440	0.003060
5	2	4.24	0.003358	0.001642	0.003858
6	2	7.81	0.003538	0.001462	0.004038
7	2	22.46	0.004047	0.000953	0.004547
1	3	0.00	0.000000	0.005000	0.0000001
2	3	0.56	0.002005	0.002995	0.002005
3	3	1.11	0.002470	0.002530	0.002470
4	3	2.19	0.002975	0.002025	0.002975
5	3	4.24	0.003050	0.001950	0.003050
6	3	7.81	0.003115	0.001885	0.003115
7	3	22.46	0.003404	0.001596	0.003404
end					

4.2.5 Replication assays involving different salts

Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.1 M imidazole, 6 µl scale, exp.1-2: T = 10 °C, exp.3: T = r.t.

a: No Additional Salt

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.02	0.000034	0.004966	0.000034
3	1	2.15	0.000034	0.004966	0.000034
4	1	4.17	0.000091	0.004909	0.000091
5	1	7.70	0.000177	0.004823	0.000177
6	1	21.07	0.000494	0.004506	0.000494
7	1	28.82	0.000701	0.004299	0.000701
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.02	-0.00001	0.005011	0.000489
3	2	2.15	0.000067	0.004933	0.000567
4	2	4.17	0.000166	0.004834	0.000666
5	2	7.70	0.000289	0.004711	0.000789
6	2	21.07	0.000718	0.004282	0.001218
7	2	28.82	0.000901	0.004099	0.001401
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.02	0.000042	0.004958	0.000042
3	3	2.15	0.000115	0.004885	0.000115
4	3	4.17	0.000127	0.004873	0.000127
5	3	7.70	0.000293	0.004707	0.000293
6	3	21.07	0.000405	0.004595	0.000405
7	3	28.82	0.000456	0.004544	0.000456
end					

b: 0.2 M NaCl

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.07	-0.000006	0.005006	-0.000006
3	1	2.21	0.000021	0.004979	0.000021
4	1	4.22	0.000089	0.004911	0.000089
5	1	7.71	0.000221	0.004779	0.000221
6	1	21.19	0.000573	0.004427	0.000573
7	1	28.87	0.000686	0.004314	0.000686
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.07	0.000040	0.004960	0.000540
3	2	2.21	0.000086	0.004914	0.000586
4	2	4.22	0.000221	0.004779	0.000721
5	2	7.71	0.000393	0.004607	0.000893
6	2	21.19	0.000731	0.004269	0.001231
7	2	28.87	0.000936	0.004064	0.001436
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.07	0.000016	0.004984	0.000016
3	3	2.21	0.000034	0.004966	0.000034
4	3	4.22	0.000098	0.004902	0.000098
5	3	7.71	0.000241	0.004759	0.000241
6	3	21.19	0.000514	0.004486	0.000514
7	3	28.87	0.000566	0.004434	0.000566
end					

c: 0.2 M Na_2SO_4

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	0.94	-0.000003	0.005003	-0.000003
3	1	1.92	-0.000019	0.005019	-0.000019
4	1	3.97	0.000046	0.004954	0.000046
5	1	6.64	0.000101	0.004899	0.000101
6	1	21.89	0.000388	0.004612	0.000388
7	1	29.92	0.000536	0.004464	0.000536
1	2	0.00	0.000000	0.005000	0.000500
2	2	0.94	0.000107	0.004893	0.000607
3	2	1.92	0.000083	0.004917	0.000583
4	2	3.97	0.000154	0.004846	0.000654
5	2	6.64	0.000190	0.004810	0.000690
6	2	21.89	0.000600	0.004400	0.001100
7	2	29.92	0.000778	0.004222	0.001278
1	3	0.00	0.000000	0.005000	0.0000001
2	3	0.94	0.000005	0.004995	0.000005
3	3	1.92	0.000046	0.004954	0.000046
4	3	3.97	0.000071	0.004929	0.000071
5	3	6.64	0.000091	0.004909	0.000091
6	3	21.89	0.000245	0.004755	0.000245
7	3	29.92	0.000268	0.004732	0.000268
end					

d: 0.2 M NaI

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	0.97	0.000018	0.004982	0.000018
3	1	1.96	-0.000010	0.005010	-0.000010
4	1	4.01	0.000084	0.004916	0.000084
5	1	6.67	0.000172	0.004828	0.000172
6	1	21.89	0.000677	0.004323	0.000677
7	1	29.92	0.000905	0.004095	0.000905
1	2	0.00	0.000000	0.005000	0.000500
2	2	0.97	0.000034	0.004966	0.000534
3	2	1.96	0.000074	0.004926	0.000574
4	2	4.01	0.000210	0.004790	0.000710
5	2	6.67	0.000326	0.004674	0.000826
6	2	21.89	0.000948	0.004052	0.001448
7	2	29.92	0.001159	0.003841	0.001659
1	3	0.00	0.000000	0.005000	0.0000001
2	3	0.97	0.000021	0.004979	0.000021
3	3	1.96	0.000067	0.004933	0.000067
4	3	4.01	0.000152	0.004848	0.000152
5	3	6.67	0.000335	0.004665	0.000335
6	3	21.89	0.000465	0.004535	0.000465
end					

4.2.6 Replication assay involving 0.4 M EDC

Conditions: 5 mM tri-PNAs **A** and **B**, 0.4 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 6 µl scale, exp.1-2: T = 10 °C, exp.3: T = r.t.

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.12	0.000080	0.004920	0.000080
3	1	2.07	0.000108	0.004892	0.000108
4	1	4.14	0.000224	0.004776	0.000224
5	1	8.49	0.000574	0.004426	0.000574
6	1	22.27	0.001209	0.003791	0.001209
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.12	0.000118	0.004882	0.000618
3	2	2.07	0.000226	0.004774	0.000726
4	2	4.14	0.000412	0.004588	0.000912
5	2	8.49	0.000746	0.004254	0.001246
6	2	22.27	0.001444	0.003556	0.001944
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.12	0.000101	0.004899	0.000101
3	3	2.07	0.000197	0.004803	0.000197
4	3	4.14	0.000290	0.004710	0.000290
5	3	8.49	0.000515	0.004485	0.000515
6	3	22.27	0.000828	0.004172	0.000828
end					

comparable experiment at 0.2 M EDC

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.07	-0.000006	0.005006	-0.000006
3	1	2.21	0.000021	0.004979	0.000021
4	1	4.22	0.000089	0.004911	0.000089
5	1	7.71	0.000221	0.004779	0.000221
6	1	21.19	0.000573	0.004427	0.000573
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.07	0.000040	0.004960	0.000540
3	2	2.21	0.000086	0.004914	0.000586
4	2	4.22	0.000221	0.004779	0.000721
5	2	7.71	0.000393	0.004607	0.000893
6	2	21.19	0.000731	0.004269	0.001231
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.07	0.000016	0.004984	0.000016
3	3	2.21	0.000034	0.004966	0.000034
4	3	4.22	0.000098	0.004902	0.000098
5	3	7.71	0.000241	0.004759	0.000241
6	3	21.19	0.000514	0.004486	0.000514
end					

4.2.7 Replication assay at pH 6.6

Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS, 0.2 M NaCl, 0.1 M imidazole, 6 µl scale, exp.1-2: T = 10 °C, exp.3: T = r.t.

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.02	0.000046	0.004954	0.000046
3	1	1.98	0.000131	0.004869	0.000131
4	1	4.05	0.000312	0.004688	0.000312
5	1	8.38	0.000916	0.004084	0.000916
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.02	0.000012	0.004988	0.000512
3	2	1.98	0.000163	0.004837	0.000663
4	2	4.05	0.000546	0.004454	0.001046
5	2	8.38	0.001153	0.003847	0.001653
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.02	0.000105	0.004895	0.000105
3	3	1.98	0.000342	0.004658	0.000342
4	3	4.05	0.000583	0.004417	0.000583
5	3	8.38	0.001168	0.003832	0.001168
end					

comparable experiment at pH 7.6: see 4.2.5 b

comparable experiment at pH 7.2: see 4.2.3 b

4.2.8 Replication assay optimized for fast replication and high conversion

Conditions: 5 mM tri-PNAs **A** and **B**, 0.4 M EDC, 0.2 M MOPS pH 6.6, 0.1 M imidazole, 10 °C, 6 µl scale.

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.09	0.000185	0.004815	0.000185
3	1	2.16	0.000378	0.004622	0.000378
4	1	3.52	0.000730	0.004270	0.000730
5	1	5.16	0.001171	0.003829	0.001171
6	1	7.05	0.001647	0.003353	0.001647
7	1	23.47	0.003277	0.001723	0.003277
1	2	0.00	0.000000	0.005000	0.000250
2	2	1.09	0.000246	0.004754	0.000496
3	2	2.16	0.000537	0.004463	0.000787
4	2	3.52	0.000895	0.004105	0.001145
5	2	5.16	0.001421	0.003579	0.001671
6	2	7.05	0.001851	0.003149	0.002101
7	2	23.47	0.003310	0.001690	0.003560
1	3	0.00	0.000000	0.005000	0.000500
2	3	1.09	0.000255	0.004745	0.000755
3	3	2.16	0.000593	0.004407	0.001093
4	3	3.52	0.000965	0.004035	0.001465
5	3	5.16	0.001450	0.003550	0.001950
6	3	7.05	0.001820	0.003180	0.002320
7	3	23.47	0.003498	0.001502	0.003998
1	4	0.00	0.000000	0.005000	0.001000
2	4	1.09	0.000277	0.004723	0.001277
3	4	2.16	0.000614	0.004386	0.001614
4	4	3.52	0.001033	0.003967	0.002033
5	4	5.16	0.001603	0.003397	0.002603
6	4	7.05	0.001951	0.003049	0.002951
7	4	23.47	0.003441	0.001559	0.004441
end					

4.2.9 Replication assays involving different imidazole concentrations

Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.2, 0.2 M NaI, 10 °C, 6 µl scale.

a: 0.1 M Imidazol

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	0.87	0.000092	0.004908	0.000092
3	1	1.50	0.000106	0.004894	0.000106
4	1	3.17	0.000288	0.004712	0.000288
5	1	5.67	0.000515	0.004485	0.000515
6	1	9.12	0.000829	0.004171	0.000829
7	1	23.63	0.001792	0.003208	0.001792
1	2	0.00	0.000000	0.005000	0.000500
2	2	0.87	0.000070	0.004930	0.000570
3	2	1.50	0.000139	0.004861	0.000639
4	2	3.17	0.000361	0.004639	0.000861
5	2	5.67	0.000683	0.004317	0.001183
6	2	9.12	0.001012	0.003988	0.001512
7	2	23.63	0.002050	0.002950	0.002550
end					

b: 0.2 M Imidazole

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	0.75	0.000083	0.004917	0.000083
3	1	1.38	0.000055	0.004945	0.000055
4	1	3.05	0.000202	0.004798	0.000202
5	1	5.55	0.000383	0.004617	0.000383
6	1	9.00	0.000484	0.004516	0.000484
7	1	23.52	0.001119	0.003881	0.001119
1	2	0.00	0.000000	0.005000	0.000500
2	2	0.75	0.000075	0.004925	0.000575
3	2	3.05	0.000258	0.004742	0.000758
4	2	5.55	0.000518	0.004482	0.001018
5	2	9.00	0.000734	0.004266	0.001234
6	2	23.52	0.001301	0.003699	0.001801
end					

4.2.10 Replication assay in the eutectic phase system at -19 °C

Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, -19 °C, 12 µl scale.

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	2.10	-0.000002	0.005002	-0.000002
3	1	4.32	0.000050	0.004950	0.000050
4	1	7.20	0.000092	0.004908	0.000092
5	1	23.45	0.000392	0.004608	0.000392
6	1	30.87	0.000494	0.004506	0.000494
7	1	48.70	0.000763	0.004237	0.000763
1	2	0.00	0.000000	0.005000	0.000500
2	2	2.10	0.000057	0.004943	0.000557
3	2	4.32	0.000147	0.004853	0.000647
4	2	7.20	0.000177	0.004823	0.000677
5	2	30.87	0.000542	0.004458	0.001042
6	2	48.70	0.000831	0.004169	0.001331
end					

comparable experiment at 10 °C: see 4.2.6

4.2.11 Replication assays involving PEG

Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, 20 % PEG, 6 µl scale, exp.1-2: *T* = 10 °C, exp.3: *T* = r.t.

a: PEG400

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.15	-0.000024	0.005024	-0.000024
3	1	2.12	0.000007	0.004993	0.000007
4	1	4.17	0.000026	0.004974	0.000026
5	1	7.98	0.000091	0.004909	0.000091
6	1	21.65	0.000292	0.004708	0.000292
1	2	0.00	0.000000	0.005000	0.000700
2	2	1.15	0.000020	0.004980	0.000720
3	2	2.12	0.000052	0.004948	0.000752
4	2	4.17	0.000142	0.004858	0.000842
5	2	7.98	0.000294	0.004706	0.000994
6	2	21.65	0.000666	0.004334	0.001366
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.15	-0.000016	0.005016	-0.000016
3	3	2.12	0.000006	0.004994	0.000006
4	3	4.17	0.000070	0.004930	0.000070
5	3	7.98	0.000112	0.004888	0.000112
6	3	21.65	0.000257	0.004743	0.000257
end					

b: PEG3350

numb.	exp.	time[h]	t-t0	a	t
1	1	0.00	0.000000	0.005000	0.0000001
2	1	1.03	-0.000023	0.005023	-0.000023
3	1	2.02	0.000023	0.004977	0.000023
4	1	4.07	0.000128	0.004872	0.000128
5	1	7.88	0.000340	0.004660	0.000340
6	1	21.53	0.001081	0.003919	0.001081
1	2	0.00	0.000000	0.005000	0.000500
2	2	1.03	-0.000006	0.005006	0.000494
3	2	2.02	0.000076	0.004924	0.000576
4	2	4.07	0.000283	0.004717	0.000783
5	2	7.88	0.000572	0.004428	0.001072
6	2	21.53	0.001403	0.003597	0.001903
1	3	0.00	0.000000	0.005000	0.0000001
2	3	1.03	0.000010	0.004990	0.000010
3	3	2.02	0.000089	0.004911	0.000089
4	3	4.07	0.000249	0.004751	0.000249
5	3	7.88	0.000402	0.004598	0.000402
6	3	21.53	0.000889	0.004111	0.000889
end					

comparable experiment at 10 °C: see 4.2.6

4.2.11 Replication assay from three independent series of experiments

Conditions: 5 mM tri-PNAs **A** and **B**, 0.2 M EDC, 0.2 M MOPS pH 7.6, 0.2 M NaCl, 0.1 M imidazole, 16 µl scale, T = 10 °C.

numb.	exp.	time[h]	t	t0	a
1	1	0	0.000001	0.000001	0.005000
2	1	1	0.000019	0.000019	0.004981
3	1	2	0.000045	0.000045	0.004955
4	1	4.1	0.000117	0.000117	0.004883
5	1	6	0.000194	0.000194	0.004806
6	1	7.7	0.000255	0.000255	0.004745
7	1	9.1	0.000311	0.000311	0.004689
8	1	24.5	0.000853	0.000853	0.004147
9	1	28.4	0.000951	0.000951	0.004049
10	1	29.9	0.001011	0.001011	0.003989
1	2	0	0.000250	0.000001	0.005000
2	2	1	0.000286	0.000036	0.004964
3	2	2	0.000304	0.000054	0.004946
4	2	4.1	0.000435	0.000185	0.004815
5	2	6	0.000543	0.000293	0.004707
6	2	7.7	0.000617	0.000367	0.004633
7	2	9.1	0.000672	0.000422	0.004578
8	2	24.5	0.001267	0.001017	0.003983
9	2	28.4	0.001372	0.001122	0.003878
10	2	29.9	0.001441	0.001191	0.003809
1	3	0	0.000500	0.000001	0.005000
2	3	1	0.000549	0.000049	0.004951
3	3	2	0.000610	0.000110	0.004890
4	3	4.1	0.000719	0.000219	0.004781
5	3	6	0.000824	0.000324	0.004676
6	3	7.7	0.000911	0.000411	0.004589
7	3	9.1	0.000975	0.000475	0.004525
8	3	24.5	0.001613	0.001113	0.003887
9	3	28.4	0.001697	0.001197	0.003803
10	3	29.9	0.001774	0.001274	0.003726
1	4	0	0.001000	0.000001	0.005000
2	4	1	0.001014	0.000014	0.004986
3	4	2	0.001100	0.000100	0.004900
4	4	4.1	0.001237	0.000237	0.004763
5	4	6	0.001346	0.000346	0.004654
6	4	7.7	0.001445	0.000445	0.004555
7	4	9.1	0.001507	0.000507	0.004493
8	4	24.5	0.002170	0.001170	0.003830
9	4	28.4	0.002285	0.001285	0.003715
10	4	29.9	0.002352	0.001352	0.003648
end					